

## 報 文

## Detection of dehydrin-like proteins in buckwheat by immunoblotting with an antibody against the lysine-rich sequence

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## Abstract

Dehydrin is a group 2 LEA (late embryogenesis abundant) protein that is widely found in plants under water stress and in mature plant seeds. As yet, the presence of dehydrin in buckwheat has not been reported. In this study, we detected dehydrin-like proteins in buckwheat by using immunoblotting with an antibody against dehydrin's highly conserved lysine-rich sequence. Both major (20 kDa) and minor (16 kDa) bands were found in common buckwheat flours, as well as in dried and in cooked buckwheat noodles. By using SDS-PAGE, it was difficult to recognize the major dehydrin-like proteins, since their molecular weight is very close to that of legumin (22-24 kDa), an abundant storage protein of buckwheat. In two-dimensional electrophoresis, two spots at pI 7.2 reacted with the antibody to the dehydrin motif. The minor dehydrin-like proteins at 16 kDa showed moderate resistance to pepsin digestion. From these results, it was suggested that buckwheat and its products contain dehydrin-like proteins, which might be associated with pepsin resistance and buckwheat allergy.

Keywords: LEA protein, late embryogenesis abundant protein; PAGE, polyacrylamide gel electrophoresis; PMSF, phenylmethanesulphonyl fluoride; PVDF, polyvinylidene difluoride;

## Introduction

Buckwheat (*Fagopyrum esculentum*) is a widely grown annual crop belonging to the Polygonaceae family. It is known as a good source of protein, having an amino acid score of 92%<sup>1)</sup>. In recent years, buckwheat has become much more popular in many countries as a type of health food that prevents hypertension and cardiovascular diseases. However, ingestion of buckwheat is known to cause immediate-type anaphylactic reactions through a specific IgE antibody<sup>2,3)</sup>. The incidence of buckwheat allergy is increasing in Asia and Europe. Buckwheat is designated as one of five specific food materials that need to

be labeled as allergens by the Ministry of Health, Labour and Welfare of Japan, since the ingestion of small amounts of buckwheat sometimes provokes severe symptoms, including anaphylactic reactions<sup>4,6)</sup>. However, it remains controversial as to which protein is responsible for such reactions. Recently, Tanaka et al. reported that a pepsin-resistant 16 kDa protein is associated with immediate hypersensitivity reactions in patients who have buckwheat allergy<sup>7)</sup>. They found that this protein had a homology with rice dehydrin.

Dehydrin is a group of LEA (late embryogenesis abundant) proteins with highly conserved lysine-rich sequences. It is commonly found in plants under water stress and in mature plant seeds<sup>9)</sup>. Although scientific in-

formation is scarce, a few researchers have suggested the involvement of dehydrin proteins in food allergies. Chung et al. suggested that accumulation of dehydrin in peanuts increased IgE binding and advanced glycation end adducts (AGEs)<sup>8</sup>. There have been, however, no reports on the presence of dehydrin in buckwheat. In our previous studies, we found dehydrin proteins in soybean seed<sup>10</sup>, and extended this work to include the molecular diversity of dehydrin in soybean<sup>11, 12</sup> and to the characteristics of rice dehydrin<sup>13</sup>. In this study, we tried to detect dehydrin proteins by using an antibody for the lysine motif sequence specific to dehydrin.

## Materials and Methods

### Materials

Refined or whole grain flours of two varieties of buckwheat (cv. Kitawase from Japan and cv. Mankan from China) were kindly supplied by Dr. Horigane at the National Food Research Institute. Dried 100% buckwheat noodles (Tokyo Kajino) were purchased at a local market. Thirty grams of noodle was cooked for 6 min in 150 ml of boiling water, and settled for 2 min. The cooking water (Soba-yu) was also analyzed after centrifugation at 10,000 × g for 10 min.

### SDS-PAGE and immunoblotting analysis

SDS-PAGE was carried out following the method of Laemmli<sup>14</sup>. Proteins were extracted from flours and powdered noodles with 10 volumes (v/w) of sample buffer. Boiled noodles (300 mg) were ground in 1 ml of sample buffer. Water used for cooking (Soba-yu) was mixed with an equal volume of SDS-PAGE sample buffer at twice the concentration. All of the samples were centrifuged and boiled for 5 min. Samples were loaded on 5-20% polyacrylamide gradient gel (PAGEL, NPG-520L, ATTO), and electrophoresis was carried out at constant current of 20 mA for 80 min. Gels were either stained with Coomassie Brilliant Blue (CBB) R-250 or subjected to immunoblotting.

For the immunoblotting, gels were incubated in a blotting buffer (25 mM Tris-HCl, pH 9.5 containing 40 mM ε-aminocaproic acid, 20% methanol, and 0.05% SDS) for 20 min, and then the proteins on the gel were blotted onto a PVDF membrane (Immun-Blot, Bio Rad) at

2.5 mA for 30 min. The PVDF membrane was kept overnight in 3% BSA-TBS (Tris-Buffered Saline, 20 mM Tris-HCl, pH 7.5, 500 mM NaCl, containing 3% bovine serum albumin). Dehydrin protein was detected by using polyclonal rabbit antiserum raised against a conserved lysine-motif sequence (DQNEKKGIMDKIKEKLPGGH) conjugated with ovalbumin<sup>13</sup>. The PVDF membrane was incubated for 60 min in the first antibody diluted to 1:100 with 1% BSA-TBS, and then was washed with TBS-t (Tris-buffered saline containing 0.05% tween 20) three times for 20 min each, and with TBS for 20 min. The washed membrane was incubated with peroxidase-conjugated anti-rabbit goat antibody diluted to 1:1000 with 1% BSA-TBS, washed with TBS-t three times for 20 min, and with TBS for 20 min, and then was detected by a peroxidase detection kit (Immunostain HRP1000 Konica).

Reactivity of patients' sera to buckwheat allergens was examined with 1:10 diluted sera supplied by Kokusai Bio Co. Ltd. and 1:2000 diluted horseradish peroxidase-conjugated goat anti-human IgG antibody (Zymed Lab. Inc.) in a procedure similar to that described above.

### Two-dimensional electrophoresis

Buckwheat flour (500 mg) was mixed with 5 ml of extraction buffer (30 mM Tris-HCl, pH 7.5 containing 0.02 % sodium azide and 1 mM PMSF) and centrifuged at 10,000 × g for 20 min. Two-dimensional electrophoresis was carried out by using a Multiphore II System (GE Healthcare) following the supplier's instructions as described in a previous paper<sup>13</sup>. IPG strips (pH 3-10NL, 7 cm) were incubated overnight in a protein fraction mixed with sample buffer (3-10NL). The IPG strips were then washed with distilled water, and put onto the Multiphore system. Then electrophoresis was carried out at 200-3500 V for 90 min and at 3500 V for 65 min. After equilibration in 50 mM Tris-HCl, pH 8.8 containing 6 M urea, 30% (w/v) glycerol, 2% SDS, 10 mg/ml dithiothreitol, and a trace of bromophenol blue, SDS-PAGE was carried out on a 5-20% polyacrylamide gradient gel, and stained with CBB R-250.

### Pepsin digestion assay

The stability of water-soluble proteins from buckwheat flour was assayed by using the method of Astwood

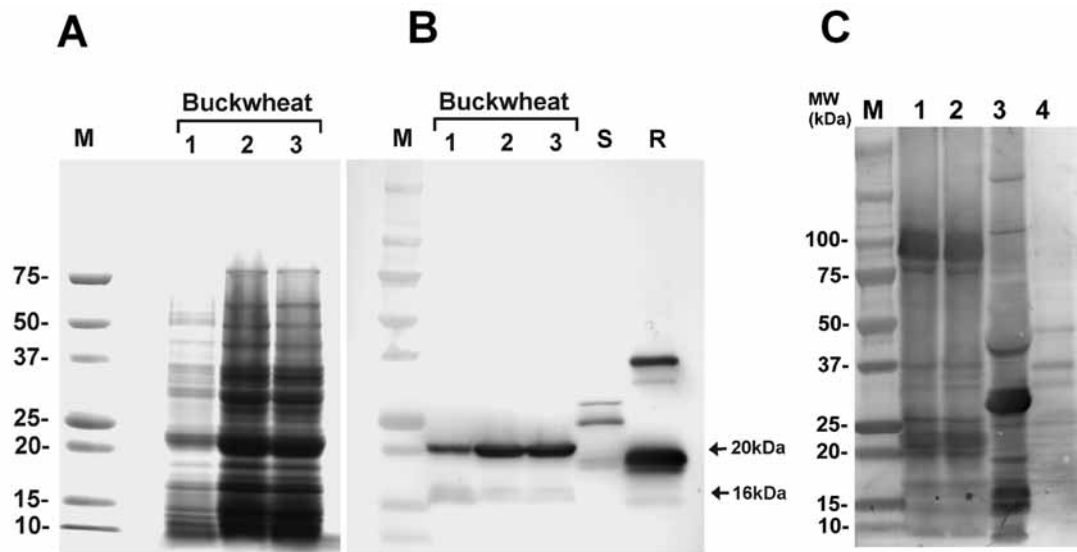


Fig. 1 Detection of dehydrin-like proteins in buckwheat flour.

A: SDS-PAGE pattern of buckwheat protein:

lanes 1 and 2, refined and whole grain flour of Chinese buckwheat (cv.Mankan); lane 3, whole grain flour of Japanese buckwheat (cv.Kitawase).

B: Immunoblotting for the lysine-rich sequence of dehydrin.

lane 1-3, same as Fig. 1A; S, soy protein; R, rice dehydrin<sup>13</sup>.

C: Immunoblotting using patients' serum.

lane 1, whole grain flour of Chinese buckwheat (cv. Mankan); lane 2, whole grain flour of Japanese buckwheat (cv. Kitawase); lane 3, protein extract from red kidney bean<sup>17</sup>, lane 4, rice dehydrin fraction<sup>13</sup>.

et al.<sup>15</sup>). Whole grain flour (cv. Kitawase) was mixed in 10 volumes (v/w) of water and homogenized in a Hiscotron homogenizer (NS50, Nichi-On) for 1 min at 10,000 rpm. The extract was centrifuged at  $8,000 \times g$  for 20 min, and then the supernatant was collected. Protein content of the extract was estimated by using the micro assay procedure for a Bradford assay reagent (Bio Rad).

## Results and Discussion

Figure 1A shows the SDS-PAGE pattern of refined and whole grain buckwheat flour prepared from Chinese and Japanese varieties. Although refined flour contained a lower amount of proteins than did whole grain flours, the SDS-PAGE profiles of the flours were similar. Both in the refined and in the whole grain flours, the most abundant protein was legumin (BW24KD), a major storage protein that is often referred to as 11S globulin. BW24KD is a frequently recognized allergenic component, binding to IgE antibodies from patients' sera<sup>16</sup>. Dehydrin-like proteins in buckwheat were detected by immunoblotting with an antibody against its highly conserved lysine-rich se-

quence (Fig. 1B). A prominent band at 20 kDa was observed in all of the buckwheat flours, and a faint band was also detected at 16 kDa. Since the molecular weight of the major dehydrin-like protein was very close to that of legumin, it was difficult to recognize it as a distinct band in the SDS-PAGE profile. Probably, these are 20 kDa dehydrin-like proteins form an adjacent band at the lower edge of that of legumin at 22-24 kDa in the SDS-PAGE.

Figure 1C shows the results of immunoblotting by using patients' sera. Though it is still controversial as to which protein (or proteins) is responsible for buckwheat allergy, 59-67, 34-37, 26, 19, and 16 kDa proteins have all been reported to be associated with buckwheat allergy<sup>6</sup>. Recent studies have verified that BW24KD is one of the main allergens in common buckwheat, but other buckwheat allergens may also be of importance (such as those of 19, 16, and 9 kDa). In this experiment, a major reaction was observed in 100 kDa and 22-24 kDa polypeptides, and several additional minor bands were detected, including a band at 20 kDa that could be attributed to dehydrin-like proteins in buckwheat flour.

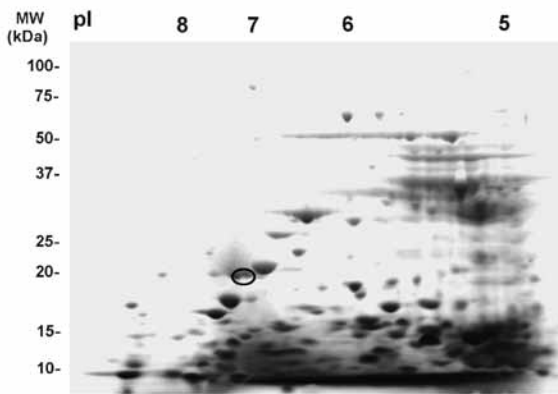


Fig. 2 Two-dimensional electrophoresis of buckwheat flour.

Circled spots reacted with the antibody to the lysine-rich sequence of dehydrin.

In two-dimensional electrophoresis of whole grain flour, two obscure spots of 20 kDa (circled in Fig. 2) were recognized separately from a major 22-23 kDa spot at pI 7. Immunoblotting analysis following electrophoresis revealed that these 20 kDa spots reacted with the antibody to the conserved lysine-rich sequence of dehydrin (data not shown).

In order to determine the effect of processing and cooking, dried noodles labeled as 100% buckwheat were purchased and cooked following the manufacturer's instructions. The water used for boiling (Soba-Yu) was also analyzed, since the water is usually consumed after eating the noodles. The dried commercial noodles, the cooked noodles, and the cooking water (Soba-Yu) all showed similar SDS-PAGE profiles (Fig. 3A). In the results of immunoblotting, major dehydrin-like proteins (20 kDa) were found in the dried and cooked noodles as well as in the cooking water (Fig. 3B). This result indicated that substantial amounts of dehydrin-like proteins were present in popular buckwheat food; boiled noodles and Soba-Yu. The faint band of dehydrin-like proteins at 16-18 kDa was not detected in the cooking water. Typically, dehydrin shows heat tolerance, remaining soluble after several minutes of boiling. The 16-18 kDa proteins appeared to lack heat stability.

In general, food allergens are considered to be stable to various digestive treatments. As mentioned earlier, Tanaka et al. found a pepsin-resistant 16 kDa protein associated with an immediate hypersensitivity reaction to

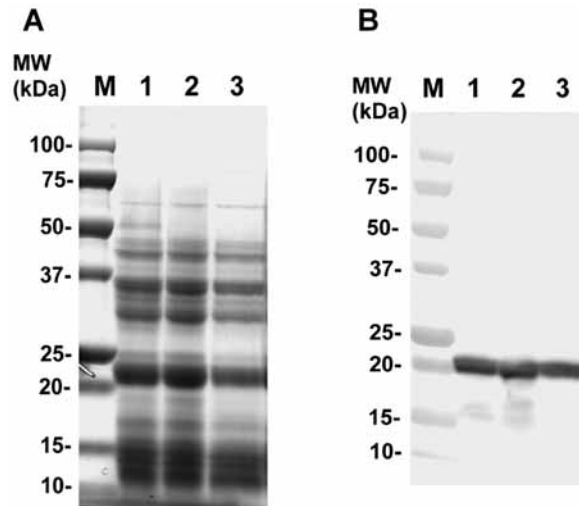


Fig. 3 Detection of dehydrin-like proteins in buckwheat noodles and in cooking water.

lane 1, dried noodles; lane 2, cooked noodles; lane 3, cooking water (Soba-Yu)

buckwheat allergens. They found that the *N*-terminal sequence of the 16 kDa protein (RDEGFDLGETQMSSK) had 80% homology with *Oriza sativa* dehydrin (Gene Band Accession No.OSU60097)<sup>7)</sup>. Although little information exists, a few researchers have indicated that dehydrin proteins are involved in food allergy. Chung et al. suggested that accumulation of dehydrin in the peanut seed increased IgE binding and advanced glycation end adducts (AGEs)<sup>8)</sup>. Wang et al. reported the heat stability of a 24 kDa allergenic protein in tartary buckwheat seed<sup>18)</sup>. The pepsin digestion assay was conducted to examine the digestibility of dehydrin-like proteins found in the buckwheat flour (Fig. 4). Proteins with high molecular weight, including legumin (22-24 kDa), the major protein in buckwheat, were promptly digested within 1 to 2 min. Low-molecular weight polypeptides increased in 1 to 8 min digestion, and decreased thereafter. Figure 4B shows the immunoblotting detection of the dehydrin motif in the digests. The 20 kDa dehydrin-like protein was digested within 1 min, while the 18 kDa polypeptide, which appeared to be a partial digestion product of the 20 kDa dehydrin-like protein, was detected from 25 s to 1 min of digestion. The 16kDa protein showed moderate tolerance to pepsin digestion. It is of interest whether this 16 kDa dehydrin-like protein is related to the 16 kDa protein that Tanaka et al. and other groups have found associated with buckwheat allergy<sup>6,7)</sup>.

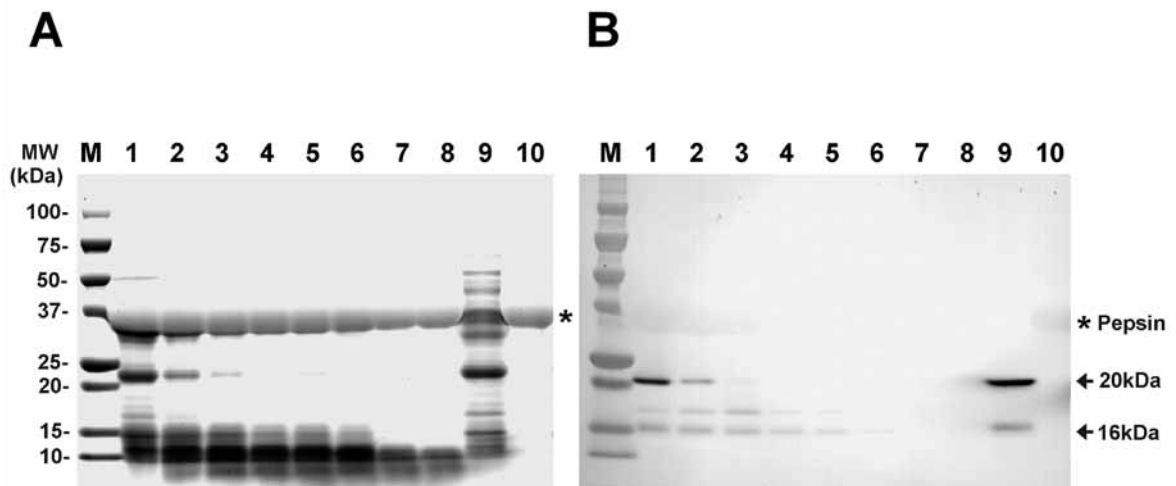


Fig. 4 Pepsin digestion assay of buckwheat protein.

A: CBB-stained gel.

B: Immunoblotting of the lysine-rich sequence of dehydrin.

Extracts from whole grain flour were incubated in simulated gastric fluid composed of 0.32% pepsin from porcine stomach (3300 units/mg, Wako), 30 mM NaCl, pH 1.2, for 0, 0.25, 1, 2, 4, 8, 15, and 60 min (lanes 1-8). Buckwheat protein and SGF were run alone in lanes 9 and 10, respectively.

In conclusion, we found major (20 kDa) and minor (16 kDa) dehydrin-like proteins in common buckwheat flours, and in dried and cooked buckwheat noodles by using immunoblotting analysis with antibodies against highly conserved lysine-rich sequences of dehydrin. This is the first report to recognize dehydrin proteins in buckwheat. In SDS-PAGE results, it was difficult to recognize the major dehydrin-like proteins, since their molecular weight was very close to that of legumin (22-24 kDa), a major allergen protein of buckwheat. Two-dimensional electrophoresis enabled us to separate the major dehydrin-like proteins from legumin. Although the minor dehydrin-like proteins at 16 kDa showed moderate resistance to pepsin digestion, further studies are needed to investigate whether or not this protein is associated with pepsin resistance and buckwheat allergy.

#### Acknowledgment

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